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MEK-1 activates C-Raf through a Ras-independent mechanism

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ABSTRACT

C-Raf is a member of the Ras–Raf–MEK–ERK mitogen-activated protein kinase (MAPK) signaling pathway that plays key roles in diverse physiological processes and is upregulated in many human cancers. C-Raf activation involves binding to Ras, increased phosphorylation and interactions with co-factors. Here, we describe a Ras-independent *in vivo* pathway for C-Raf activation by its downstream target MEK. Using ^{32}P-metabolic labeling and 2D-phosphopeptide mapping experiments, we show that MEK increases C-Raf phosphorylation by up-to 10-fold. This increase was associated with C-Raf kinase activation, matching the activity seen with growth factor stimulation. Consequently, coexpression of wildtype C-Raf and MEK was sufficient for full and constitutive activation of ERK. Notably, the ability of MEK to activate C-Raf was completely Ras independent, since mutants impaired in Ras binding that are irresponsive to growth factors or Ras were fully activated by MEK. The ability of MEK to activate C-Raf was only partially dependent on MEK kinase activity but required MEK binding to C-Raf, suggesting that the binding results in a conformational change that increases C-Raf susceptibility to phosphorylation and activation or in the stabilization of the phosphorylated-active form. These findings propose a novel Ras-independent mechanism for activating the C-Raf and the MAPK pathway without the need for mutations in the pathway. This mechanism could be of significance in pathological conditions or cancers overexpressing C-Raf and MEK or in conditions where C-Raf–MEK interaction is enhanced due to the down-regulation of RKIP and MST2.

1. Introduction

The mammalian serine/threonine kinase family, Raf, consists of three highly conserved members: A-Raf, B-Raf and C-Raf/c-Raf-1 [1–4]. Raf is part of the phosphorylation cascade of the *mi*togen-*a*ctivated *p*rotein *k*inase (MAPK) pathway, Ras–Raf–MEK–ERK [5–7]. The MAPK pathway is involved in the regulation of a large variety of cellular physiological functions and its uncontrolled activation leads to cell transformation [8]. Importantly, increased activation of the MAPK pathway is seen in more than 50% of all human cancers, either through the abnormal activation of growth factor receptors or through mutations in the pathway itself. For example, 30% of all human cancers carry an activating Ras mutation while many others carry activating mutations in the B-Raf gene, predominantly in melanomas and thyroid cancers [9–11].

C-Raf activation involves multiple protein–protein interactions and phosphorylation events that are not yet fully understood [1,4]. The initial step following stimulation of cells with various growth factors includes the accumulation of the active, GTP-bound Ras, which binds and recruits cellular inactive C-Raf to the plasma membrane. Two C-Raf domains mediate the binding of GTP-Ras to C-Raf: a Ras-binding domain, RBD, amino acids 57–131 and a zinc finger cysteine-rich domain, CRD, amino acids 139–184 (Fig. 1) [1,12–14]. The binding to Ras is thought to enforce a conformational change in C-Raf, opening the molecule to phosphorylation and other potential activating sequences at the plasma membrane [1,4,15]. Following activation, C-Raf dissociates from Ras and can be found in the cytoplasm associated with several scaffold proteins including the regulatory protein, 14-3-3, which is required for maintaining the active state of C-Raf [16,17].

Phosphorylation of C-Raf during the activation process is critical for conferring the active conformation and mutation of key phosphorylation sites or their dephosphorylation inactivates C-Raf kinase activity. Of the known C-Raf phosphorylation sites, phosphorylations at S338/9 and Y340/1, located in a conserved regulatory region, and phosphorylations at S471 and S491/4, located in the catalytic domain (Fig. 1), are thought to have the most significant role in C-Raf activation

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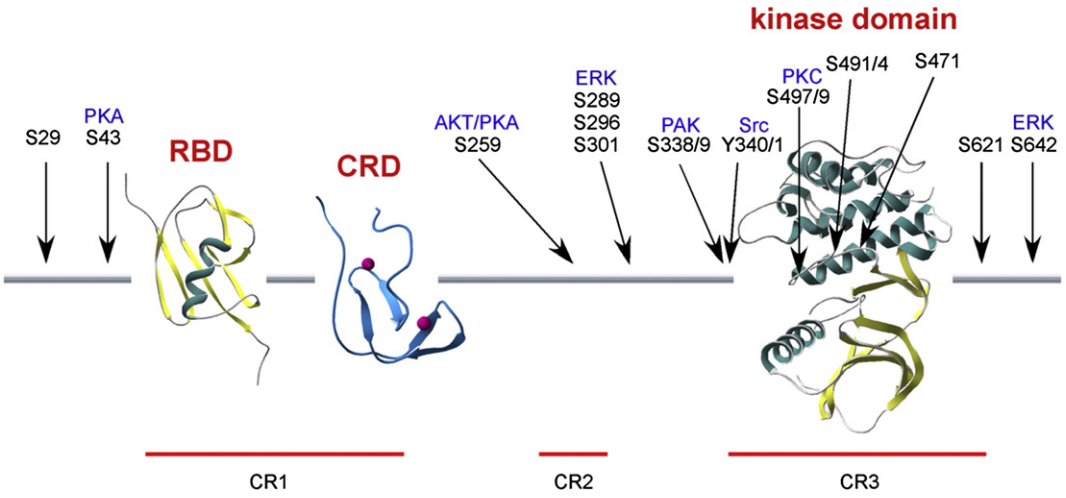

Fig. 1. C-Raf domains and known phosphorylation sites. Indicted are the C-Raf phosphorylation sites and the kinases that have been reported to phosphorylate these sites, the kinase domain and the Ras binding domains: RBD (Ras binding domain) and CRD (cysteine rich domain). CR1, 2 and 3 are conserved regions among the Raf family members. See text for further details.

[1–4,18,19]. Another key phosphorylation site is at S621, mediating the interaction of C-Raf with the 14-3-3 proteins [15,20]. Several of the other identified phosphorylation sites seem to play a negative role in C-Raf regulation, including S43 and S259, which can be phosphorylated by PKA and AKT [1,4].

Besides the conventional, Ras-induced C-Raf activation, several reports also propose Ras-independent C-Raf activation modes. For example, deletion of the N-terminal regulatory region that contains the Ras binding domains results in an increased C-Raf kinase activity [21,22]. This form appears naturally as a viral-transmitted oncogene, however, the fact that it can be further activated by growth factor stimulation, suggests that modifications within the catalytic region itself are involved in the normal activation process [23]. Another example is the ability of PAK and Src family kinases to partially activate C-Raf in a Ras independent manner by phosphorylating an auto-inhibitory C-Raf regulatory region containing S338 and Y341 sites [24,25].

Protein kinase C family members, including PKC δ were reported initially as Ras-independent C-Raf activators by showing that phorbol esters can activate C-Raf in a manner not inhibited by a dominant negative Ras form, Ras N17 [26,27]. However, a later study demonstrated that mutations within the RBD domain completely abolished C-Raf activation by phorbol esters [28]. Since phorbol esters strongly activate Ras, it is possible that the dominant negative Ras was not sufficient in blocking the Ras-induced C-Raf activation. In addition, mutations of the proposed PKC phosphorylation sites of C-Raf, S497/499, do not affect C-Raf activation by growth factors or PMA [29].

During the initial elucidation of the MAPK pathway, early studies reported C-Raf phosphorylation by ERK *in vitro*, but the functional significance of this phosphorylation was not determined [30,31]. Later studies that established the signaling direction from Raf to MEK and from MEK to ERK, diverted the focus from these initial observations [5,32], however, they have been confirmed in more recent studies demonstrating that ERK phosphorylates C-Raf at multiple sites, including S289, 296, 301 and S642, providing a feedback regulatory mechanism [33,34]. Controversy remains, however, as to whether this phosphorylation serves as a positive or negative regulation [1,4]. Our work points to a positive regulation of C-Raf by ERK-mediated phosphorylation by stabilizing the active form of C-Raf [34]. It is important to note, however, that the ERK-mediated C-Raf phosphorylation does not activate C-Raf or enhances its activation by itself, but it rather prolongs the activation duration induced by growth factors.

MEK-1 was also reported to positively regulate C-Raf: Alessandrini et al. [35] showed that constitutively active forms of MEK-1 can induce the transformation of NIH 3T3 and Swiss 3T3 cells and that clonal cell lines expressing active MEK-1 mutants displayed elevated C-Raf kinase activity. Confirming these results, Zimmermann et al. [36] reported that a constitutively active MEK-1 and to a lesser extent, wildtype MEK-1, can induce increased C-Raf phosphorylation. This increased phosphorylation correlated with C-Raf activation (2-fold with wildtype MEK and 6-fold with active MEK). The ability of active MEK to induce C-Raf activation was not dependent on Ras or Src, but was dependent on ERK kinase activity, suggesting a positive feedback loop mediated by ERK.

Here, we provide conclusive evidence that MEK-1 is a potent, Ras-independent C-Raf activator. Our data demonstrate that coexpression of wildtype MEK-1 and C-Raf induces a strong increase in C-Raf phosphorylation, resulting in full activation of C-Raf kinase activity, similar to that achieved with growth factors. Interestingly, the increased C-Raf phosphorylation was due primarily to an increase in the basal phosphorylation sites. The ability of MEK to induce C-Raf phosphorylation and activation required C-Raf binding and kinase activity, but was only partially dependent on MEK-1 kinase activity itself. Importantly, wildtype MEK was able to fully activate C-Raf mutants impaired in Ras binding and was not affected by a dominant negative Ras mutant, demonstrating complete independence of Ras. We propose that direct interaction of C-Raf and MEK-1 contributes to C-Raf activation by inducing C-Raf hyper-phosphorylation, either by conferring a conformation that is more susceptible to phosphorylation or by stabilizing the phosphorylated form of C-Raf. These findings have potential significance in cancers that overexpress wildtype C-Raf and MEK or in cases where RKIP [37] or MST2, which are natural inhibitors of C-Raf–MEK interaction, are down-regulated.

2. Materials and methods

2.1. cDNA constructs, antibodies and kinase inhibitors

The bacterial expression vectors pGEX-GST–MEK-1 and GST-ERK-1 and the mammalian expression vectors pExchange 5a-FLAG-MEK-1, pMT2-HA-ERK-1, pMT2-myc-C-Raf expressing wildtype or S471A/T, S259/621A, S289/296/301A mutants and pCMV5-FLAG-Ras N17 or V12 were described previously [12,15,18,34,38]. The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to generate pMT2-myc-C-Raf mutants containing the following mutations: S338/339A, K84A/L86A/K87A (Ras binding domain, RBD mutant), C165/168S and C165/168A (cysteine rich domain, CRD mutants), K375M (kinase-dead, KD mutant) and their combinations: RBD/CRD, RBD/KD and RBD/CRD/KD. pExchange 5a-FLAG-MEK-1 was used as a

template for generating a kinase-dead mutant (K97M), a proline-rich domain deletion (Δ 265–301) and DVD mutants (W374D, L375E and WL374/375DE). Antibodies for MEK and ERK and phosphospecific antibodies for their active forms were from Cell Signaling Technology (Beverly, MA), FLAG antibody was from Sigma (St. Louis, MO) and antibodies for HA and myc epitope tags were produced from the 12CA5 and 9E10 hybridoma clones, respectively. Phosphospecific antibodies for C-Raf pS296 were produced and screened for specificity as described previously [34]. The MEK inhibitor U0126 was from Promega (Madison, WI).

2.2. Cell culture and transfection

COS-7 cells were maintained in DMEM supplemented with 10% fetal calf serum. For transient expression of proteins, cells were transfected using LipofectAMINE (Invitrogen, Carlsbad, CA) as detailed in the figure legends according to the manufacturer's instructions. For cell stimulation, 24 h after transfection, cells were deprived of serum for 18 h before adding the agonist. Details for cell stimulation and drug treatments are provided below and in the figure legends.

2.3. ^{32}P metabolic labeling

Serum-deprived COS-7 cells transfected as indicated in the figure legends were washed once with medium lacking phosphate, followed by 30-min incubation in the phosphate-free media for depleting intracellular phosphate. Cells were radio-labeled by incubation in the presence of 0.5 mCi/ml ^{32}P for 2 h as previously described [34].

2.4. Cell extraction and protein purification

Cells were lysed for 30 min using ice-cold extraction buffer containing 50 mM Tris–Cl, pH 7.5, 100 mM NaCl, 1% Triton X 100, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 50 mM ß-glycerophosphate and a protease inhibitor cocktail (Amersham, Piscataway, NJ). For immunoprecipitation, cleared cell lysates were incubated at 4 °C for 2 h with the appropriate antibody precoupled to protein A/G agarose-beads (SantaCruz, Santa Cruz, CA). The beads were washed twice with extraction buffer, twice with extraction buffer containing 0.5 M LiCl and twice with kinase assay buffer (40 mM Tris–Cl pH 7.5, 0.1 mM EDTA, 5 mM $MgCl_2$ and 2 mM DTT). The purified proteins were used further as specified in the figure legends.

2.5. C-Raf phosphopeptide mapping

Two-dimensional phosphopeptide mapping was performed according to previously described protocols [39,40]. Briefly, immunopurified ^{32}P-labeled myc-C-Raf proteins were resolved using 7.5% SDS-PAGE, transferred to a PVDF membrane, excised, and ^{32}P incorporation in myc-C-Raf was determined by Cherenkov counting. Following incubation with 0.5% poly-vinyl-phosphate (PVP) in 100 mM acetic acid for 30 min at 37 °C and extensive washes, C-Raf protein samples were digested with 10 μg sequencing grade modified trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate buffer for 2 h at 37 °C and with additional 10 μg of trypsin for overnight (this method routinely allowed recovery of 90–95% of the initial radioactivity in myc-C-Raf). The eluted peptides were washed twice with 50 mM ammonium bicarbonate buffer and once with pH 1.9 TLC-electrophoresis buffer (2.2% Formic acid and 7.8% acetic acid in water). Samples were spotted on cellulose TLC plates (Merck, Darmstadt, Germany) and separated using the Hunter thin-layer chromatography system (CBS Scientific, Del Mar, CA) in pH 1.9 buffer for 25 min at 1000 V. The plates were dried overnight and subjected to second dimension chromatographic separation in a phospho-chromatography buffer (37.5% n-Butanol, 25% Pyridine and 7.5% acetic acid). The plates were dried and the phosphopeptide spots were visualized by autoradiography and phosphor-imaging. Each experiment was repeated at least three times and representative maps are presented.

2.6. C-Raf kinase assay

C-Raf kinase activity was determined using a slight modification of a previously described protocol [18,34]. Briefly, following myc immunoprecipitation, myc-C-Raf containing beads were incubated in kinase assay buffer (100 μl final volume) supplemented with 100 μM ATP, and 0.4 μg prokaryotic recombinant GST–MEK-1-His for 30 min at 30 °C. Samples were separated on 10% SDS-PAGE and transferred to PVDF membranes. The phosphorylation of MEK-1 was determined by phospho-MEK-immunoblotting or using ^{32}P ATP in the kinase reaction and densitometry quantification. In some cases, Raf kinase activity was determined in a coupled kinase assay, where 1 μg GST–ERK was added to the kinase reaction and incubated for additional 30 min as previously described [18]. myc-C-Raf recovery was determined by myc-immunoblotting. Each kinase experiment was repeated at least three times and representative kinase experiments are presented.

3. Results

3.1. Coexpression of wildtype C-Raf and MEK-1 results in increased C-Raf phosphorylation and kinase activity

In the course of studying C-Raf–MEK-1 interactions, we noticed that coexpression of C-Raf with MEK-1 results in a significant mobility shift in C-Raf, suggesting that MEK-1 could induce increased C-Raf phosphorylation [18]. To test this possibility, we examined the effect of MEK-1 on C-Raf phosphorylation using ^{32}P-metabolic labeling and 2D phospho-peptide mapping of C-Raf (Fig. 2). Surprisingly, MEK-1 expression induced a much stronger C-Raf phosphorylation than the one observed with growth factors, reaching up to a 10-fold increase in overall C-Raf phosphorylation (Fig. 2a, compare lanes 1, 2 and 11 and Fig. 2b, lanes 1, 2 and 5). To examine whether this increase was due to new phosphorylation sites or due to an increase in the basal sites, C-Raf phosphorylation was analyzed using 2D phosphopeptide mapping (Fig. 2b). Note that we loaded equal counts of trypsin-digested C-Raf peptides from the various samples in this analysis (1500–2000 cpm) in order to be able to compare the relative stoichiometry of the various phosphorylation sites. Therefore, the MEK-induced increases in overall C-Raf phosphorylation do not appear in the maps. This analysis revealed that the MEK-induced increase in C-Raf phosphorylation derived primarily from an increase in the basal C-Raf phosphorylation sites rather than from phosphorylation at new sites (Fig. 2b, compare panels 1 and 5). Although we observed several new C-Raf phosphorylation sites in the MEK-1 coexpressing cells (Fig. 2b, panel 9, spots n, x, y and z), these sites accounted only for a small fraction of the overall C-Raf phosphorylation. Importantly, MEK-1 did not induce the phosphorylation of C-Raf S471A and S471T mutants that abrogate MEK-1 binding [18] (Fig. 2a, compare lanes 1 and 11 (wildtype C-Raf) with lanes 3 and 9 (S471A) or with lanes 5 and 10 (S471T) and Fig. 2b, compare lanes 1 and 5 (wildtype) with lanes 3 and 6 (S471A)). This result indicates that MEK-1 binding to C-Raf is required for the enhanced C-Raf phosphorylation, suggesting that MEK-1 may confer a C-Raf conformation that is more accessible to phosphorylation or is protected from dephosphorylation. In addition, C-Raf activity itself was not required for the ability of MEK-1 to induce C-Raf phosphorylation, since the increase in phosphorylation was also observed with an inactive C-Raf mutant, C-Raf S259/621A [15] (Fig. 2b, compare lanes 7 and 8). Similarly, MEK kinase activity also appeared to play a minor role, since treatment with the MEK inhibitor U0126 had a small effect on the enhanced C-Raf phosphorylation (Fig. 2a, compare lanes 8 and 11). Overall, these experiments support the notion that MEK-1 increases

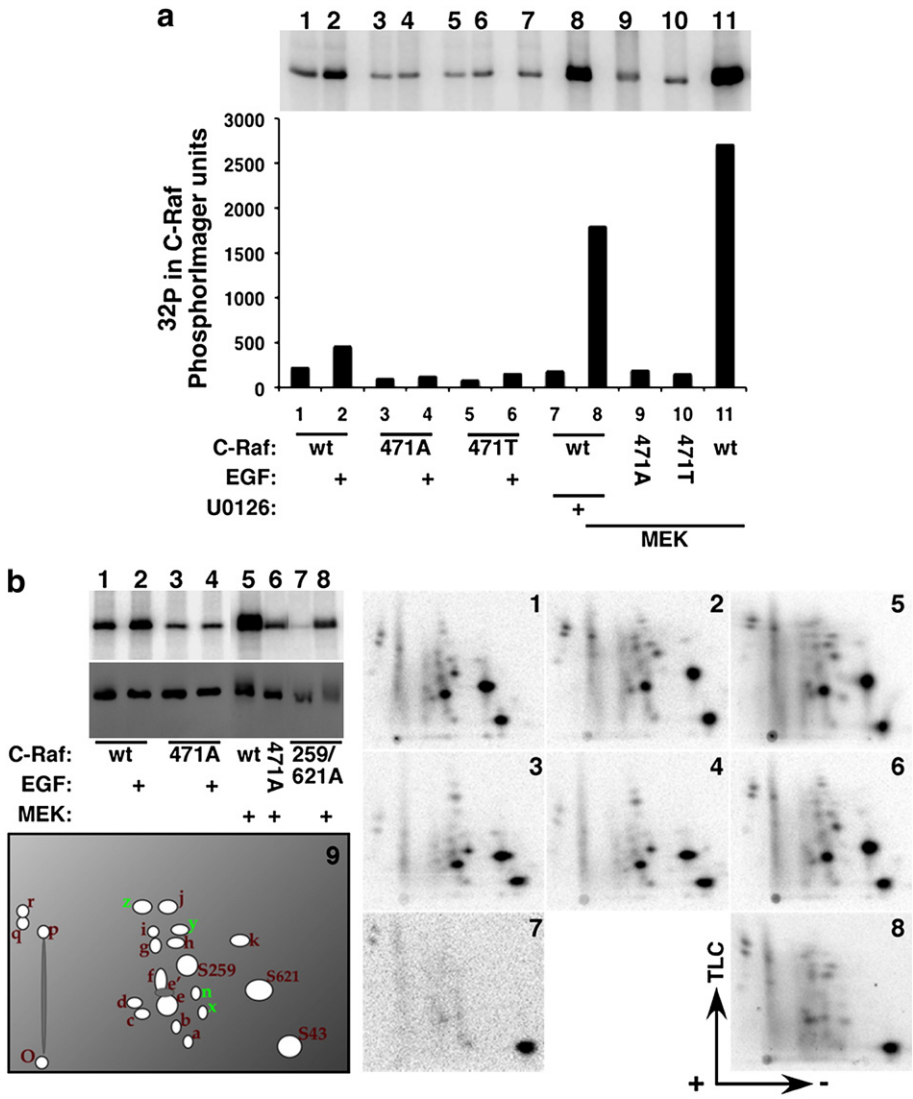

Fig. 2. MEK-1 expression induces increased C-Raf phosphorylation. (a) COS-7 cells were transfected with pMT2-myc-C-Raf wildtype (wt), S471A or S471T mutants alone (lanes 1–7) or together with pExchange 5a-FLAG-MEK-1 (lanes 8–11). Following 24 h, cells were deprived of serum for 18 h, metabolically labeled with ^{32}P and treated with vehicle or 100 ng/ml EGF for 30 min. Indicated samples (lanes 7 and 8) were treated with 20 μM of the MEK inhibitor, U0126, 30 min prior to ^{32}P-labeling. myc-C-Raf proteins were immunoprecipitated with a myc-epitope tag antibody and separated using SDS-PAGE. ^{32}P incorporation in myc-C-Raf was visualized using a phosphor imager (inset) and quantified using the Bio-Rad Quantity One software. (b) Cells were transfected as indicated and the samples were analyzed as in a (left top panel). The ^{32}P bands were excised from the membrane and 2D phosphopeptide mapping was performed as described in Materials and methods (panels 1–8). 10% of the sample was used to examine myc-C-Raf recovery (bottom part). A schematic representation of the phosphopeptide spots, including the migration positions of known C-Raf phosphopeptides are depicted in panel 9. Arrows indicate the orientation of electrophoresis and TLC chromatography. Note that equal amounts of counts were loaded from each sample (1200 cpm, excluding sample 7) to allow comparison of phosphorylation stoichiometry between the samples. For sample 7, S259/621A mutant, only 300 cpm was available since this mutant is consistently less phosphorylated than the other C-Raf forms. The results are representative of three independent experiments.

C-Raf phosphorylation through direct binding to C-Raf, independent of C-Raf or MEK-1 kinase activities.

Since C-Raf phosphorylation has been correlated with its kinase activity, we examined next the effect of MEK-1 expression on the intrinsic kinase activity of C-Raf (Fig. 3). In these experiments, myc-epitope-tagged C-Raf purified from serum-deprived COS-7 cells expressing C-Raf alone or from cells coexpressing C-Raf with MEK-1 was assayed *in vitro* for kinase activity using recombinant bacterially-expressed GST–MEK-1 as a substrate. Surprisingly, coexpression of MEK-1 was sufficient to induce same extent of C-Raf activation in serum-deprived cells as the one achieved with EGF stimulation (Fig. 3, compare lanes 2 and 3). We did not observe much additive effect of EGF and MEK-1 on C-Raf activation (Fig. 3, lane 4). We showed previously a positive feedback regulation of C-Raf by ERK-mediated C-Raf phosphorylation [34]. Notably, in this work, ERK did not activate c-Raf *per se* and it did not enhance the basal kinase activity of C-Raf in unstimulated cells, rather, it was able to prolong the activation duration of C-Raf in cells stimulated by growth factors. To examine the role of ERK in the MEK-induced C-Raf activation, we used a mutant of C-Raf, S289/296/301A, lacking the ERK phosphorylation sites and also coexpressed ERK with MEK and C-Raf (Fig. 3). ERK overexpression did not affect the extent of C-Raf activation by either EGF or MEK (Fig. 3, compare lanes 1–4 and 5–8). However, the S289/296/301A C-Raf mutant showed decreased activation by MEK compared to wildtype C-Raf (Fig. 3, compare lanes 3 and 11), suggesting that the ERK-induced phosphorylation and/or these sites play some role in the MEK-induced C-Raf activation process.

Together, these results point to MEK-1 as a novel C-Raf activator, which is able to activate C-Raf in the absence of growth factors and as efficiently as the conventional growth factor receptor-Ras pathway, possibly by directly binding C-Raf and increasing its basal phosphorylation. Of note, incubation of C-Raf purified from serum-deprived cells

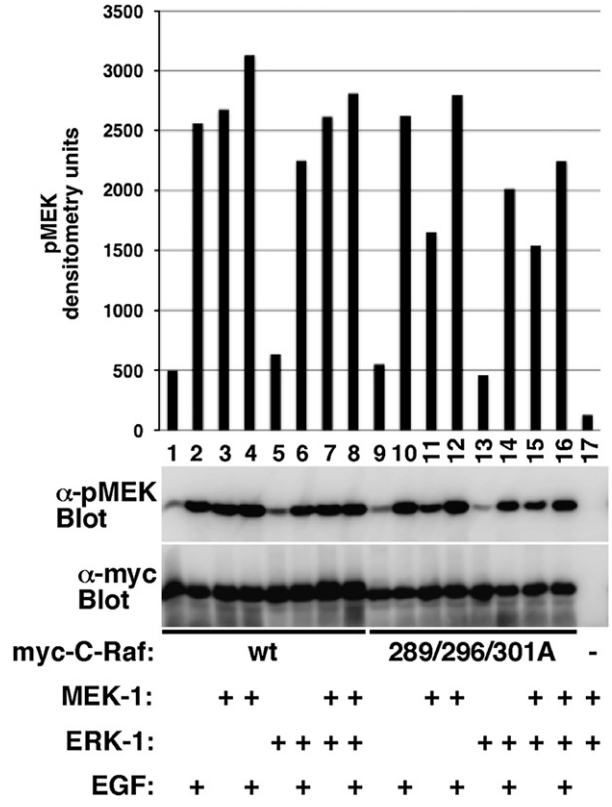

Fig. 3. MEK-1 expression increases C-Raf kinase activity. COS-7 cells were transfected with pMT2-myc-C-Raf (wt) or S289/296/301A mutant alone or together with pExchange 5a-FLAG-MEK-1 or pMT2-HA-ERK1 as indicated. After 24 h, cells were deprived of serum for 18 h and treated with vehicle or 100 ng/ml EGF for 20 min. C-Raf kinase activity in myc-immunoprecipitates was assayed using recombinant GST–MEK-1 as a substrate as described in Materials and methods. Presented are phospho-MEK and myc immunoblots showing MEK phosphorylation and myc-C-Raf recovery, respectively. The bar graph shows densitometry quantification of the phospho-GST–MEK-1 band. Presented are representative results of three independent experiments.

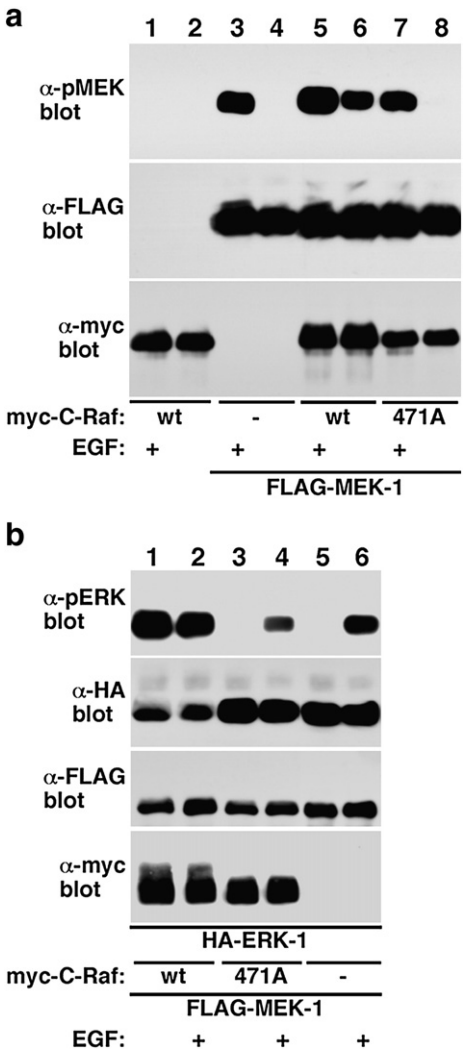

Fig. 4. Coexpression of MEK-1 and C-Raf induces constitutive activation of MEK and ERK. (a) COS-7 cells were transfected with wildtype pMT2-myc-C-Raf (wt, lanes 1, 2 and 5, 6), the S471A mutant (471A, lanes 7, 8) or empty pMT2 vector (lanes 3, 4), alone (lanes 1, 2) or together with pExchange 5a-FLAG-MEK-1 (lanes 3–8). After 24 h, cells were deprived of serum for 18 h and treated with vehicle or 100 ng/ml EGF for 20 min as indicated. MEK-1 activation in cells was determined following FLAG immunoprecipitation and immunoblotting with phospho-MEK (top panel). MEK-1 recovery was examined by FLAG-immunoblotting (middle panel) and myc-C-Raf expression was determined in cell extracts by myc immunoblotting (bottom panel). (b) COS-7 cells expressing the above C-Raf and MEK-1 expression vectors, as indicated, together with pMT2-HA-ERK-1 vector were stimulated with EGF as in a. ERK phosphorylation was determined following HA immunoprecipitation (top panel). ERK recovery was determined using HA immunoblotting. MEK and Raf expressions were determined in cell lysates using FLAG and myc immunoblotting respectively. Presented are representative results of five independent experiments.

with MEK *in vitro* did not activate its kinase activity [18,34], suggesting that the MEK-induced C-Raf activation is dependent on cellular factors that promote C-Raf activation *in vivo*.

3.2. Coexpression of wildtype C-Raf and MEK-1 results in constitutive activation of the ERK–MAPK pathway

To examine the cellular consequences of the ability of MEK-1 to induce C-Raf activation, we examined the phosphorylation of MEK-1 and ERK on their activation sites in COS-7 cells (Fig. 4). In these experiments, FLAG-epitope-tagged MEK-1 was coexpressed with wildtype or the S471A C-Raf mutant that does not bind MEK-1 and MEK-1 phosphorylation was determined by immunoblotting with activation-specific phospho-MEK antibodies (Fig. 4a). C-Raf expression with MEK-1 resulted in MEK-1 phosphorylation, reaching almost same levels as achieved with EGF stimulation (Fig. 4a, compare lanes 3, 4 and 5, 6). The inactive S471A C-Raf mutant, that does not bind MEK, did not induce MEK phosphorylation (Fig. 4a, compare lanes 6 and 8), indicating that both C-Raf kinase activity and MEK binding are required. In complementary experiments, we used HA-ERK-1 as readout for MEK activity, showing that coexpression of wildtype C-Raf and MEK-1 induces strong ERK-1 phosphorylation (Fig. 4b, compare lanes 1, 2 with 5, 6). Also in these experiments, the S471A C-Raf mutant was unable to activate the pathway (compare lanes 1, 2 with 3, 4).

To determine the extent of C-Raf/MEK overexpression needed to achieve constitutive activation of the pathway, increasing amounts of C-Raf, MEK-1 or ERK-1 were expressed in COS-7 cells and the activation of the pathway was determined in cells (Fig. 5a) and *in vitro* (Fig. 5b and c). These experiments showed sensitivity to small increases in C-Raf expression that were sufficient to induce full ERK phosphorylation when MEK-1 was coexpressed (Fig. 5a, compare lanes 1–5 and 6–10 or 11–15). MEK-1 by itself did not activate ERK (Fig. 5a, lanes 16 and 33). Similar results were obtained when C-Raf activity was measured *in vitro* (Fig. 5b and c, lanes 1–6). However, maximal C-Raf activation *in vitro* was reached only with maximal MEK-1 expression, suggesting lower sensitivity of the *in vitro* assay. As seen in Fig. 3, ERK overexpression did not alter the extent of C-Raf activation by MEK (Fig. 5b, compare lanes 1–6 with 7–12 and 13–18). These experiments have been repeated in several other cell lines including NIH3T3, HEK293 and the immortalized prostate

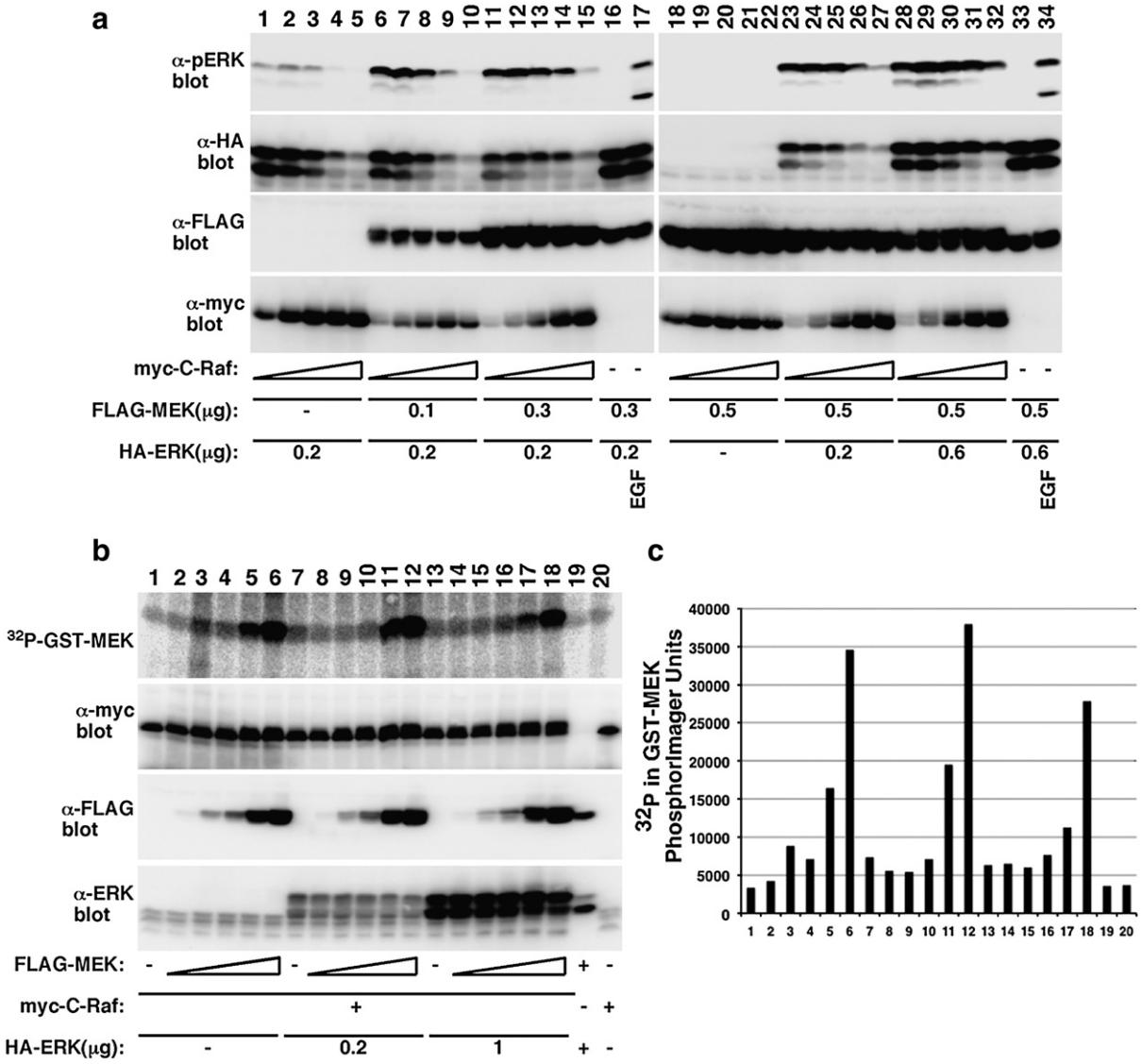

Fig. 5. Small increases in MEK-1 and C-Raf expression are sufficient for ERK activation. (a) COS-7 cells were transfected with the indicated amounts of pExchange 5a-FLAG-MEK-1 and pMT2-HA-ERK-1 together with increasing amounts of pMT2-myc-C-Raf (0.01–1 μg/plate). After 24 h, cells were deprived of serum for 18 h and treated with vehicle or 100 ng/ml EGF for 20 min, where indicated. ERK-1 activation in cells was determined by immunoblotting for phospho-ERK (top panel). Expression of myc-C-Raf, FLAG-MEK and HA-ERK was examined by myc, FLAG and HA immunoblotting, respectively. Note that increased expression of myc-C-Raf resulted in decreased HA-ERK expression. This was probably due to DNA interference since both use the pMT2 backbone. Empty pMT2 vector was used to adjust for total DNA amounts in these transfections. (b and c) COS-7 cells were transfected with pMT2-myc-C-Raf and the indicated amounts of pMT2-HA-ERK-1 together with increasing amounts of pExchange 5a-FLAG-MEK-1 (0.01–1 μg/plate). After 24 h, cells were deprived of serum for 18 h and C-Raf kinase activity was examined *in vitro* in myc immunoprecipitates using recombinant GST–MEK-1 as a substrate and ^{32}P-ATP (b, top panel). myc-C-Raf recovery, MEK and ERK expressions are also provided (b). Quantification of GST–MEK phosphorylation is provided in c. Presented are representative results of three independent experiments.

epithelial cells RWPE, all showing similar results (data not shown), pointing to the universality of this mechanism. These results demonstrate that constitutive activation of the Raf–MEK–ERK pathway can be achieved without a mutation in the pathway, but simply by co-overexpression of wildtype MEK and C-Raf.

3.3. MEK-1-induced C-Raf activation is Ras independent

To examine the role of Ras in the MEK-1-induced C-Raf activation, we used C-Raf mutants impaired in Ras binding. These included the Ras-binding domain (RBD) mutant, C-Raf K84A/L86A/K87A that impairs binding to Ras switch I region, the cysteine-rich domain (CRD) mutant, C-Raf C165/168S that impairs binding to farnesylated-Ras and a double, RBD/CRD mutant. These mutants were previously shown to be completely inactive in cells treated with various growth factors, when coexpressed with active Ras or when activation was induced by dimerization [12,29,38]. To examine the activation of the Ras-binding C-Raf mutants by MEK-1 in cells, the mutants were coexpressed in COS-7 cells with MEK-1 and their activation was determined by immunoblotting with phospho-specific MEK-1 antibodies (Fig. 6a). Both the RBD and CRD C-Raf mutants, either native or membrane-targeted by the CaaX motif, were able to induce MEK phosphorylation to the same extent as wildtype C-Raf or EGF stimulation (Fig. 6a, compare lane 3 with 4, 5 and lane 6 with 7, 8). As noted in previous studies, the CaaX forms have higher intrinsic catalytic activity than wildtype C-Raf forms.

To further examine the Ras-independent activation of the MAPK pathway in cells, ERK-1 was coexpressed with the Ras-binding C-Raf mutants and their effect on ERK-1 phosphorylation was determined (Fig. 6b). These experiments confirmed the ability of the RBD/CRD C-Raf mutants to fully activate the cascade when coexpressed with MEK (Fig. 6b, lanes 1–13). Importantly, full activation of the pathway

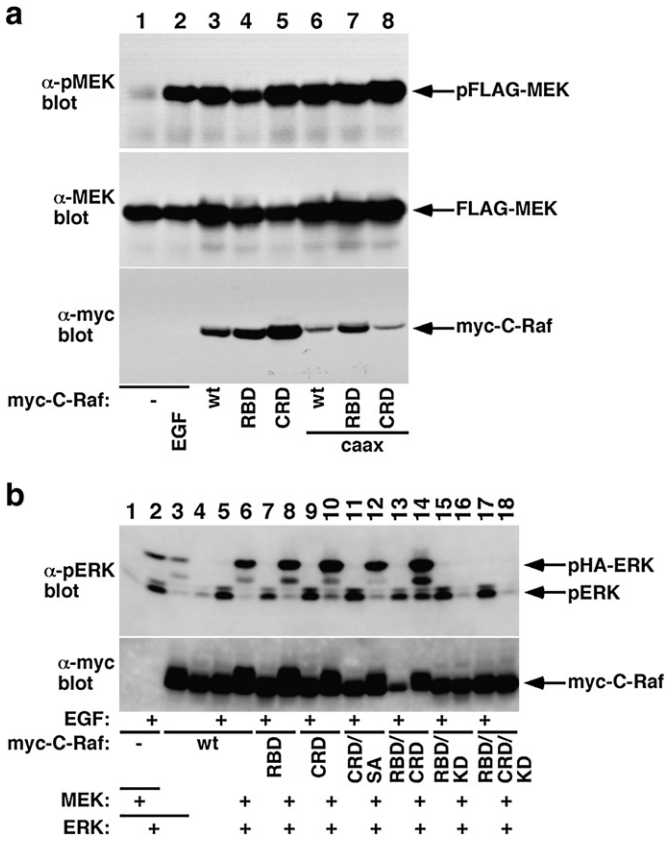

Fig. 6. MEK-induced C-Raf activation is Ras-independent. (a) COS-7 cells were transfected with pExchange 5a-FLAG-MEK-1 alone (lanes 1,2) or together with pMT2-myc-C-Raf (wt) or K84A/L86A/K87A (RBD, Ras binding domain), C165/168S (CRD, cysteine-rich domain) C-Raf mutants or with same C-Raf forms containing a c-terminal CaaX motif for membrane targeting (caax). After 24 h, cells were deprived of serum for 18 h and treated with vehicle or with 100 ng/ml EGF for 20 min as indicated. Presented are a pMEK immunoblot of FLAG-immunoprecipitates, a MEK immunoblot showing MEK recovery and a myc immunoblot showing the expression of the myc-C-Raf variants. (b) COS-7 cells were transfected as in a with the indicated DNA combinations of MEK, C-Raf variants and pMT2-HA-ERK. Presented are a pERK immunoblot in cell extracts and a myc immunoblot showing the expression of the myc-C-Raf variants. CRD/SA is C165/168A mutant, RBD/KD and RBD/CRD/KD are RBD and RBD/CRD mutants, respectively, that include also the K375M mutation in the ATP binding pocket that abrogates C-Raf kinase activity (see materials and methods for more details). Presented are representative results of four independent experiments.

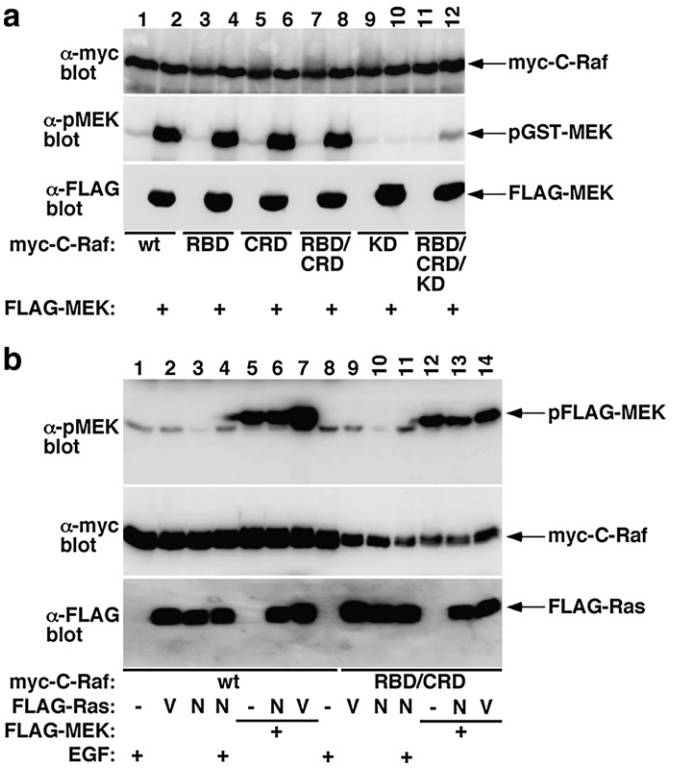

Fig. 7. Additive C-Raf activation by MEK and Ras. (a) COS-7 cells were transfected with pMT2-myc-C-Raf (wt) or K84A/L86A/K87A (RBD), C165/168S (CRD), K375M (KD) or their indicated combinations, alone or with pExchange 5a-FLAG-MEK-1 as indicated. After 24 h, cells were deprived of serum for 18 h and C-Raf kinase activity was determined in myc-immunoprecipitates using an *in vitro* kinase assay with recombinant GST–MEK as a substrate. Presented are a myc immunoblot showing myc-C-Raf recovery (top panel) pMEK immunoblot of the GST–MEK substrate (middle panel) and FLAG immunoblot of cell extracts showing FLAG-MEK expression in the cells (bottom panel). (b) COS-7 cells were transfected with pMT2-myc-C-Raf (wt) or the RBD/CRD mutant together with pExchange 5a FLAG-MEK-1 and pCMV5-FLAG-Ras V12 (V, constitutively active) or N17 (N, inactive mutant) as indicated. After 24 h, cells were deprived of serum for 18 h and treated with vehicle or with 100 ng/ml EGF for 20 min. Presented are a pMEK immunoblot of cell extracts (top panel), a myc immunoblot showing the expression of myc-C-Raf variants (middle panel) and a FLAG immunoblot showing the expression of the FLAG-Ras variants (bottom panel). The results are representative of two independent experiments.

was also observed using a CRD/RBD C-Raf double mutant, indicating complete independence from Ras binding in the MEK-induced C-Raf activation (Fig. 6b, lane 14). As an additional control, the Ras-binding mutations were introduced into the background of an ATP-binding site C-Raf mutant, K375M. This control demonstrated that the Ras-independent activation remained dependent on C-Raf kinase activity (Fig. 6b, lanes 15–18).

In addition to examining the activity of the Ras-binding C-Raf mutants in cells, we also measured their activity *in vitro* (Fig. 7a). These experiments confirmed the *in situ* experiments and demonstrated that MEK-1 can fully activate the Ras-binding C-Raf mutants (Fig. 7a, compare lane 2 with 4, 6, 8), indicating that C-Raf activation by MEK overexpression does not require direct C-Raf interaction with Ras. The K375M mutation eliminated the observed kinase activity also in this assay.

As a complementary approach to examine the role of Ras in the MEK-1-induced C-Raf activation, we used the dominant negative and constitutively active Ras forms, Ras N17 and Ras V12, respectively. In these experiments, COS-7 cells were coexpressed with MEK-1 and C-Raf along with Ras N17 or Ras V12 and their effects on MEK-1 phosphorylation in the cell were examined (Fig. 7b). The dominant negative Ras N17 had no effect on the ability of either wildtype C-Raf or the RBD/CRD C-Raf mutant to induce MEK-1 phosphorylation (Fig. 7b, compare lanes 5 and 12 with 6 and 13). Importantly, however, the active Ras V12 cooperated with MEK for wildtype C-Raf activation, but had no effect on the double RBD/CRD C-Raf mutant (Fig. 7b, compare lanes 7 and 14). This experiment suggests that MEK and Ras may be utilizing parallel independent pathways for C-Raf activation.

3.4. MEK-1-induced C-Raf activation is not dependent on MEK kinase activity

To examine the role of MEK-1 kinase activity in the MEK-1-induced C-Raf activation, we tested the effects of an inactivating MEK-1 mutation, K97M, on the ability of MEK-1 to activate C-Raf (Fig. 8). These experiments demonstrated that the kinase-dead MEK-1 mutant induced similar C-Raf activation to the one seen with wildtype MEK-1 (Fig. 8a, compare lanes 13, 14 with 15, 16). As expected, the kinase inactive MEK-1, though being phosphorylated by C-Raf, did not result in ERK activation (Fig. 8a, pERK blot, compare lanes 9, 10 with 11, 12). Also when assayed *in vitro*, the kinase-dead MEK-1 mutant induced similar C-Raf activation as achieved with wildtype MEK-1 (Fig. 8b, compare lane 1 with 7), confirming that MEK-1 activity plays only a minor or no role in the MEK-1-induced C-Raf activation.

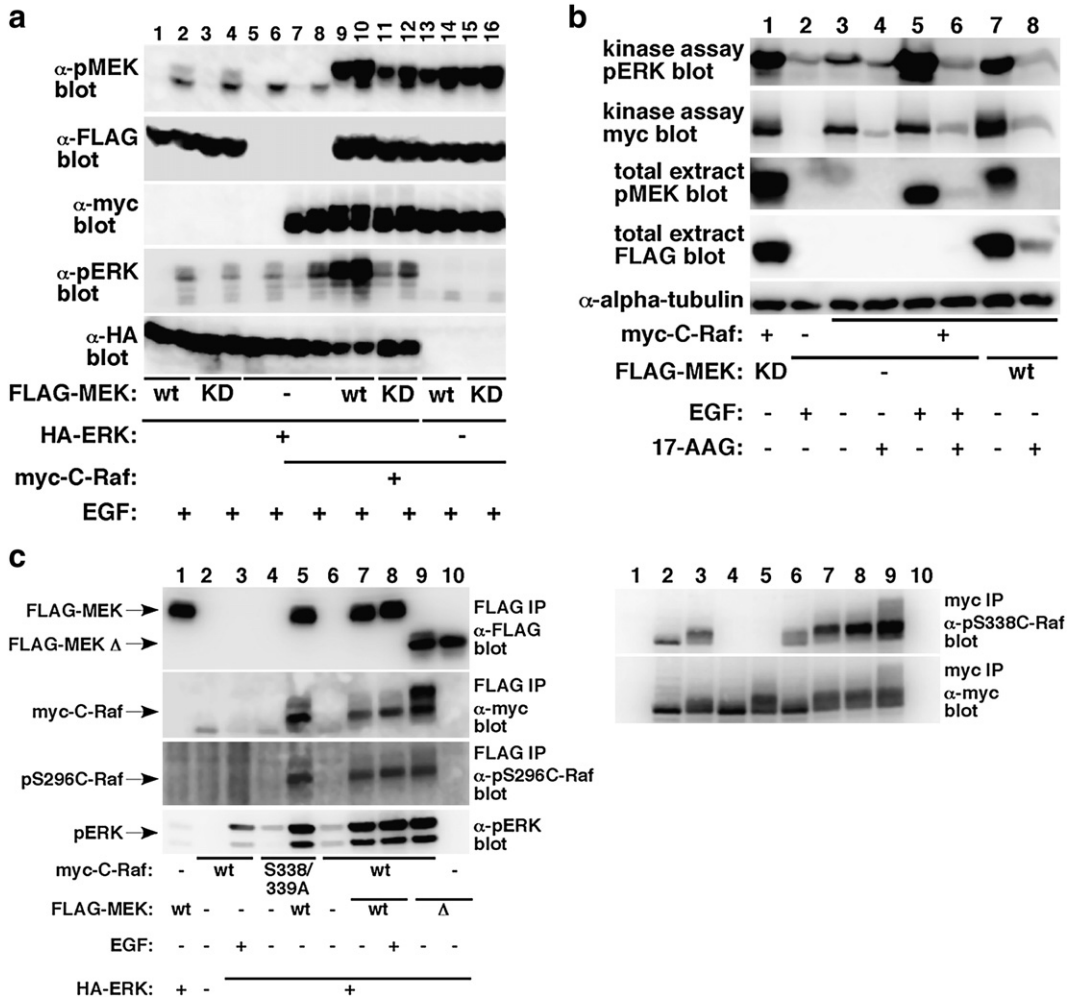

Fig. 8. MEK kinase activity is not required for the MEK-induced C-Raf activation. (a) COS-7 cells were transfected with pMT2-myc-C-Raf together with pMT2-HA-ERK-1 and pExchange 5a-FLAG-MEK-1 wildtype (wt) or a kinase dead mutant (KD, K97M) as indicated. After 24 h, cells were deprived of serum for 18 h and treated with vehicle or 100 ng/ml EGF for 20 min. Presented are phospho-MEK and phospho-ERK immunoblots of cell lysates showing MEK and ERK phosphorylation, respectively and FLAG, HA and myc-immunoblots showing FLAG-MEK-1, HA-ERK-1 and myc-C-Raf expression. (b) COS-7 cells were transfected with pMT2-myc-C-Raf together with pExchange 5a-FLAG-MEK-1 (wt, lanes 7, 8) or K97M mutant (KD, lane 1) as indicated. After 24 h, cells were deprived of serum for 18 h in the presence or absence of the heat-shock protein 90 inhibitor 17-AAG (1 μM) and treated with vehicle or 100 ng/ml EGF for 20 min. C-Raf kinase activity was measured *in vitro* in myc-immunoprecipitates using a coupled kinase assay with recombinant ERK-1 serving as a substrate. Presented are phospho-ERK and myc immunoblots of the kinase reaction showing Raf kinase activity and myc-C-Raf recovery and phospho-MEK and FLAG immunoblots of cell extracts showing MEK phosphorylation and expression in cells. Alpha-tubulin immunoblot shows equal protein loading. Note that 17-AAG treatment results in reduced myc-C-Raf and FLAG-MEK protein expressions (lanes 4, 6, 8) as has been previously reported. (c) COS-7 cells were transfected with pMT2-myc-C-Raf (wt) or S338/339A mutant together with pMT2-HA-ERK-1 and pExchange 5a-FLAG-MEK-1 wildtype (wt) or a proline-rich domain deletion mutant (Δ, deletion of amino acids 265–301) as indicated. After 24 h, cells were deprived of serum for 18 h and treated with vehicle or 100 ng/ml EGF for 20 min. Following protein extraction, samples were split and half was used to immunoprecipitate MEK using FLAG antibody (left part) and half to immunoprecipitate Raf using myc antibody (right part). In the left part, presented are FLAG, myc and pS296 C-Raf immunoblots in FLAG-immunoprecipitates (FLAG-IP), showing FLAG-MEK, myc-C-Raf and phospho-S296 C-Raf recoveries, respectively, and a pERK immunoblot of cell extracts, showing ERK phosphorylation in cells. In the right part, presented are pS338 C-Raf and myc immunoblots in myc-immunoprecipitates (myc-IP), showing C-Raf phosphorylation at the S338 site and myc-C-Raf recovery. Note that the Δ 265–301 MEK-1 mutant activates and binds C-Raf as efficiently as full-length MEK-1.

The HSP-90 inhibitor, 17-AAG, shown previously to destabilize C-Raf and to inhibit its kinase activity [41] was also able to destabilize and inhibit C-Raf coexpressed with MEK-1 (Fig. 8b, lane 8), suggesting that MEK-1 does not replace the need for interaction with HSP-90.

The lack of a role for MEK kinase activity in C-Raf activation combined with the observation that phosphorylation of the C-Raf S471A mutant that does not bind MEK-1 was unaffected by coexpression with MEK-1 (Fig. 2), led us to hypothesize that MEK-1 could be enhancing C-Raf phosphorylation and C-Raf activity through direct binding. To address this point, we attempted to generate MEK-1 forms impaired in C-Raf binding. Several previous reports claimed the identification of the C-Raf binding point on MEK. The reported sites include a proline-rich domain spanning amino acids 265–301 of MEK-1 [42], and a short domain defined as a domain for versatile docking (DVD), comprising amino acids 358–382 of MEK-1, that was suggested to be a universal domain mediating MAP2K binding to MAP3Ks [43]. Unfortunately, after generating a MEK-1 265–301 deletion as well as MEK-1 DVD mutants (W374D, L375E and WL374/375DE), which were previously shown to eliminate MEK phosphorylation by active Raf and mitogens and examining their binding to C-Raf, we found that these mutants bound and activated C-Raf as potently as wildtype MEK-1 (Fig. 8c and data not showed). For example, FLAG-MEK-1 265–301 deletion mutant when coexpressed with myc-C-Raf, induced similar ERK phosphorylation as full-length MEK-1. The deletion mutant was also able to co-immunoprecipitate equal amounts of C-Raf as full-length MEK-1 (Fig. 8c, compare lanes 6, 7 and 9). Control FLAG immunoprecipitation from cells expressing only myc-C-Raf, did not recover a detectable myc-C-Raf signal (Fig. 8c, lanes 2, 3 and 6). Interestingly, C-Raf recovered in the MEK-1 immunoprecipitates was phosphorylated at the S296 site we previously showed to be present on active C-Raf [34]

(Fig. 8c, lanes 5 and 7–9). In addition, though MEK-1 was able to induce C-Raf phosphorylation at the S338 site, which was shown to play a role in C-Raf activation by certain growth factors [19], MEK-1 was able to bind and activate the S338/339A C-Raf mutant, suggesting that the MEK-1-induced C-Raf activation does not require phosphorylation at these sites (Fig. 8c, lanes 4, 5, left panel and lanes 6–9, right panel).

4. Discussion

C-Raf is subjected to tight regulation in the cell, however, its increased activation is common in many cancers and is associated with cellular transformation [1]. Many studies have linked upregulated C-Raf activity in cancers with the presence of activating Ras mutations or increased activation of tyrosine-kinase receptors, especially EGF receptor family members [8]. Ras has been established as a primary activator of C-Raf and alternative pathways for C-Raf activation have not been widely accepted by the scientific community. In addition, overexpression of C-Raf is not considered by-itself tumorigenic, since it does not result in C-Raf activation or activation of its downstream mediators, MEK and ERK [3,22,44]. The present study provides compelling evidence supporting the existence of an alternative pathway for C-Raf activation that does not involve Ras. Our results demonstrate that elevated coexpression of wildtype C-Raf and MEK-1 in cells results in increased C-Raf phosphorylation, leading to enhanced C-Raf kinase activity, which in turn activates the downstream effectors MEK and ERK. Our data also unequivocally show that the MEK-induced C-Raf activation is completely Ras independent, requires intact S471 and S621 phosphorylation sites, but not the S338/339 sites. In addition, this activation is only partially dependent on MEK kinase activity and does not require new transcription or translation (data not showed). Importantly, modest increases in MEK and C-Raf coexpression are sufficient for maximal activation of ERK *in vivo*, suggesting clinical relevance in pathological conditions where MAPK activation is not associated with Ras mutations or over-activation of tyrosine kinase receptors.

Several studies examining C-Raf and MEK expressions in clinical samples noted increased expression in cancer specimens. For example, increased C-Raf expression has been documented in acute myeloid leukemia (AML) [45], primitive neuroectodermal tumors (PNETs) [46], non-small cell lung cancer (NSCLC) [47], ovarian cancer [48], thyroid [49] and gliomas [49]. In addition, though early studies asserted that overexpression of wildtype C-Raf or MEK is not tumorigenic, more recent work indicates that targeted overexpression of wildtype C-Raf in the lung could lead to lung cancer [50–54], suggesting that overexpression of wildtype C-Raf is sufficient for inducing cellular transformation. Our findings are of special significance since they imply that mutations along the pathway may not be necessary for constitutive pathway activation, but rather co-overexpression of C-Raf with MEK could suffice. Since initial reports indicated that overexpression of native C-Raf or MEK alone did not lead to constitutive activation of the MAPK pathway or cell transformation, few studies have looked for overexpression of Raf or MEK proteins in human cancers. Our report calls for reevaluating this premise and for a detailed examination of Raf and MEK expression levels in human cancers.

As noted in the Introduction, the current view of C-Raf activation does not give much weight to mechanisms that do not involve Ras. Indeed, besides the work by Zimmermann et al. [36], there have not been much credible or undisputed studies demonstrating C-Raf activation independent of Ras. The study by Zimmermann et al., though providing substantial evidence for such a pathway through MEK, surprisingly has not attracted enough attention in the field to establish this observation as a novel alternative mode for C-Raf activation and has not been followed up by confirming studies that further detail this novel activation mechanism. The results presented in the present study provide a more comprehensive description and analysis of this novel mechanism and help in establishing this mode as a novel path for C-Raf activation. In addition, the work by Zimmermann et al. pointed to ERK as the potential mediator of the activation, however, our results indicate that the activation is largely independent of MEK and ERK kinase activities, but rather may involve direct association of MEK with C-Raf, leading to enhanced C-Raf phosphorylation through increased susceptibility to phosphorylation or protection from dephosphorylation.

A couple of recent studies also proposed Ras-independent activation of ERK through the conventional Raf–MEK pathway, however, the exact mechanism of Raf activation has not been determined in these studies [55,56].

As noted above, the relevance of MEK ability to activate C-Raf and the MAPK pathway could be of physiological importance in pathological conditions that show increased MEK and C-Raf expression or alternatively, in cases that have decreased expression of the tumor suppressor, Raf kinase inhibitor protein, RKIP, which is a prevalent phenomenon in prostate [57] as well as several other cancers, such as thyroid, colorectal and pancreatic cancers as well as in myeloid leukemias [58–62]. RKIP is a natural inhibitor of MEK–Raf interaction, functioning by direct binding to C-Raf and blocking MEK binding [37]. Increased expression of RKIP leads to inhibition of MAPK activation and RKIP down-regulation increases MAPK activation. In natural conditions, there is not much C-Raf–MEK association, suggesting that the two proteins are kept separate. However, when we co-overexpressed C-Raf and MEK, they were easily co-purified, suggesting that their segregation was overcome by the overexpression. A recent unpublished work from Walter Kolch (personal communication) demonstrated that MST2 may function in a similar way to RKIP by blocking Raf–MEK binding and that RASSF1A regulates this axis. We propose a model where C-Raf and MEK activation can be achieved by overexpression of the proteins or alternatively, by decreased expression of RKIP or MST2, or increased expression of RASSF1A, in a Ras independent manner (Fig. 9). Thus, in cancers that have decreased expression of RKIP or MST2, the MAPK pathway may be active in a Ras independent manner and the Raf–MEK interaction could be considered for therapeutic intervention. This point is of special importance since recent studies indicate fast resistance development to Raf kinase targeting therapies [63–66].

C-Raf can be found in complex with several co-factors and adapter proteins such as 14-3-3, HSP-70 and HSP-90, scaffolds such as KSR and can also be found as homo- or hetero-dimers with B-Raf [1–3,67]. The present study adds MEK-1 as an additional C-Raf interacting protein that besides serving as a substrate also serves as a positive regulator of C-Raf, augmenting its phosphorylation and activity.

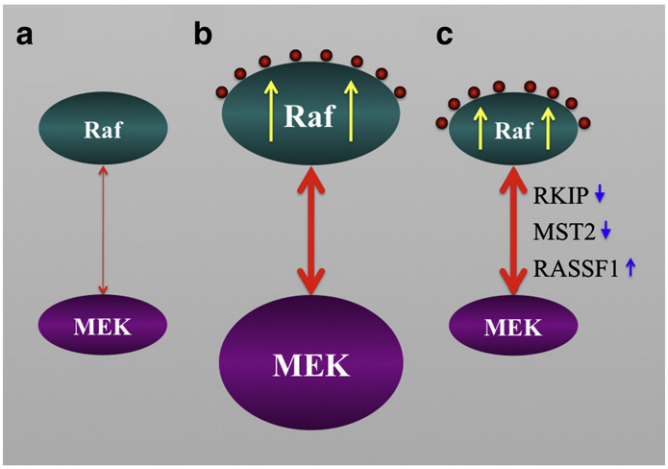

Fig. 9. A model for Ras-independent C-Raf–MEK activation. In normal conditions C-Raf and MEK expressions are low and they are maintained apart by cellular factors such as RKIP and MST2 (a). In certain conditions, such as cancer, increased expression of Raf and MEK (b) or down-regulation of the sequestering factors (c) allows increased C-Raf–MEK binding, resulting in C-Raf hyper-phosphorylation and activation.

Albeit intensive studies of C-Raf and its regulation for the past three decades, the mechanistic aspects of C-Raf association with its partners, its dynamics and regulation, with the exception of Ras and the 14-3-3 proteins, is not well established. For example, the sites that mediate C-Raf homo- or the hetero-dimerization with B-Raf, have not been determined, limiting the understanding of the functional role of Raf dimerization in its regulation [3,68,69]. In this context, a study by Rushworth et al., using overlapping peptide libraries corresponding to the entire C-Raf and B-Raf sequences, identified numerous Raf-derived peptides that can bind full-length B- and C-Raf, suggesting that there are many binding points that contribute to Raf homo- and hetero-dimerization [69]. The binding of C-Raf with MEK is even less studied. Our previous work identified C-Raf S471 as a critical site for MEK binding [18] and the analog site in A-Raf was shown later to mediate the binding of A-Raf with MEK [70]. There may be, however, other sites on Raf that contribute to MEK binding that remains to be identified. In addition, there are no predictions or proposed ideas for the nature of the Raf–MEK complex either alone or in conjunction with other complex components. Though several sites on MEK have been reported to mediate the binding to C-Raf, namely the proline rich domain [42] and the DVD domain mentioned above [43], our results indicate that deletion of the proline rich domain and mutations at the DVD domain of MEK-1 do not have an effect on C-Raf binding or the ability of MEK to activate C-Raf.

While the present work does not establish the exact mechanism by which MEK activates C-Raf, the data suggests that it involves increased C-Raf phosphorylation. It remains to be determined, however, whether this increased phosphorylation involves stabilization of the phosphorylated form by protecting C-Raf from dephosphorylation or by increasing its accessibility to kinases and phosphorylation. Also, generating MEK-1 mutants that actually block interaction with C-Raf could help in determining the role of MEK–C-Raf interaction in the MEK-induced C-Raf activation. This point is of special significance since our data point to minimal role of MEK kinase activity in this process.

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